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Atty. Docket No.: 25436/1712 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

#10

Application of:	Alan L. Greener, et al.	Examiner:	Marvich, M.
Serial No.:	09/894,806		
Filed:	June 28, 2001	Group Art Unit:	1636
Entitled:	ROOM TEMPERATURE STABLE COMPETENT CELLS	Conf. No.:	4116

Commissioner for Patents
Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. 1.131

I declare:

1. I, James F. Jolly, am an inventor of the invention claimed in the above-noted U.S. Patent application.

2. I have read and understood the Office Action mailed August 13, 2002 and have read and understood the cited reference, U.S. Patent Application No. US 2002/0081565 of Barnea et al. filed October 9, 2001, claiming priority to a provisional application filed October 30, 2000 ("Barnea et al"). I understand that the Examiner has cited the Barnea et al. application as a novelty reference over claims 1-3, 6-17, 21, 24, 29, 43, 45 and 46.

The Barnea et al. reference is cited as teaching a method for generating storage stable competent cells that are also transformed with exogenous DNA. The Office Action states that Barnea et al. teaches that cells were lyophilized at 0°C for 10 hours, 5°C, 10°C, 15°C, 20°C and 25°C for 30 minutes each in CB-I buffer with sucrose or trehalose, and that the cells exhibit a transformation efficiency of at least 10^5 transformants/μg DNA. The Office Action thus concludes that the Barnea et al. reference anticipates the claimed invention.

3. Prior to the October 30, 2000 filing date of the Barnea et al. reference, co-inventor Alan Greener and I had conceived of and reduced to practice the invention as claimed in claims 1-3, 6-17, 21, 24, 29, 43, 45 and 46. The attached Exhibit 1 consists of copies of entries from my notebooks and those of Latha Sundar, who worked under the direction of Dr. Greener and myself, that gave rise to the claimed invention. The dates on this exhibit have been redacted.

4. The ability to produce storage-stable competent cells that do not require ultra-low freezer temperatures or even standard freezer or refrigerator storage can provide great benefits to those who depend upon ready sources of competent cells. Similarly, storage stable competent cells that do not require cold shipment, e.g., with costly and cumbersome dry ice or ice shipping materials, will reduce the cost of shipping cells and the possibilities for loss of viability of the cells during shipment. It is particularly important to provide ways to render competent cells storage stable, as opposed to ways to render non-competent cells storage stable, because competent cells are generally more fragile than cells that have not been treated to make them competent. Greater care is currently required for the shipment and storage of competent, as opposed to non-competent cells.

Prior to October 30, 2000, Alan Greener and I conceived and reduced to practice a novel approach to overcome this problem. In this approach, competent cells are dried at a temperature greater than freezing, in fact, the cells need not necessarily ever be frozen in the inventive drying process, and drying can be performed at a temperature at or above room temperature. In one aspect of this approach, competent cells are dried in the presence of a water soluble glass-forming matrix, for example, a saccharide (preferably a non-reducing sugar), sugar alcohol, polyol or polymer such as polyvinylpyrolidine. In one aspect, the glass transition temperature of the cell/glass-forming matrix mixture is at least 15°C, but can be higher, e.g., higher than room temperature, such that the material is in a glass form at room temperature. Competent cells dried in the manner described in the specification remain viable and competent for at least one month at temperatures greater than –80°C, and can remain viable and competent for transformation for at least one month at temperatures greater than –20°C, 0°C, 4°C, or even room temperature or above. Such cells retain competence of at least 10^5 cfu/μg DNA upon reconstitution from such storage, and can retain higher degrees of competence, e.g., 10^6 to 10^{10} cfu/μg DNA or higher.

The attached notebook entries (Exhibit 1) demonstrate the conception and reduction to practice of the claimed invention. These experiments were performed before October 30, 2000.

A) The first notebook entry (A) from my notebook describes the preparation of Electrocompetent cells and their desiccation at an initial temperature of 4°C, ramping up to 30°C.

The notebook states:

"Electrocomp. cells

Electrocomp. cells were prepared following protocol provided by Latha S."

The entry details, in steps 1-16, the preparation of electrocompetent cells. Then, on the second page, the entry states

"Cell Dry Down

1. 40 µl aliquots of trehalose-prepared cells are pipeted into metal cups and placed into shelf of lyoph. equilibrated at 4°C shelf temp.

2. Following drying protocol used to dry down cells:"

{the entry then includes a print-out of the drying profile from the dryer, showing a starting setpoint "setpoint 1" as 4°C (a temperature above freezing) and 3000 mTorr, with subsequent temperatures of 10°C, 15°C, 20°C, 25°C and 30°C.}

{the following page states that "After drying, the cups are stored at RT in a dessicant chamber. Two dried cell discs are crushed into a DSC {differential scanning calorimetry} pan and a Tg value is determined." The entry shows the DCS results.}

{the following page describes the rehydration and plating of the rehydrated cells to determine viability – after laying out the calculations and experimental approach, that page states:

"Cell viability determined by Alan G. Dried cells were ~1% of control cells. The dried cells were electrocompetent."

B) The second notebook entry (B) from my notebook describes a subsequent drying run on electrocompetent cells as follows:

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Fast dry down

1) Electrocomp. cells were prepared as above (p 48) except drying was done for 2 hr at 30°C/3000 mTorr.

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C) Notebook entry (C) from Latha Sundar's notebook describes the drying of chemically competent ("chemicompetent") cells at a temperature greater than freezing as follows:

"Desiccation of XL-10 Gold (Chemicomp mutant strain)"

{steps 1-4 describe the initial and expansion cultures of XL-10 Gold E. coli cells and their centrifugation to pellet. Step 5, recited below, makes the cells competent using FSB buffer}

"5. Immed after spin stopped, decanted sup., added to 1 set of samples , added (1/4 vol) FSB → and made cells competent for ~ 18 min. on ice. *To the second set of samples, did NOT add anything – sat pellets on ice (At end of incubation, added same vol. (1/4) of 2% trehalose)."

{Steps 6, 7 and 8 describe spinning down the competent cells and washing them to remove traces of FSB buffer. The following page describes the desiccation of these competent cells}

"Desiccation of XL-10 Gold – Desiccation Expt 6"

" – 1:40 (the starting volume) of Trehalose 20% (Did not add sorbitol to this prep), and quickly aliquotted ~ 150 µl each into the 2 ml glass vials, all on ice. (Aliquotting took 12-15 mins.)
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Note that these chemicompetent cells were dried at 30°C without freezing immediately prior to the drying. Thus, before the October 30, 2000 date of the Barnea et al. reference, competent cells (both electrocompetent and chemicompetent) were dried at a temperature greater than freezing, thereby generating storage-stable competent cells. These cells were not frozen immediately prior to drying – that is, the cells were not freeze-dried.

The experiments described in notebook entries A-C represent a full reduction to practice of the claimed method of drying competent cells at a temperature above freezing before October 30, 2000.

5. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Date James F. Jolly

Serial No.: 09/894,806

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2/14/03

Date

James F. Jolly

Electrocomp cells

Electrocomp cells were prepared by following protocol provided by Zahn's:

1. Innoculate a single colony of E. C. E. coli (provided by Zahn) into 5ml of LB. Grow O/N at 37°C, shake.
2. Next morning transfer to 100ml of sterile LB & grow at 37°C to final O.D. of 0.7 ~~0.75~~
3. When O.D. reached place flask on ice for 15-30 min.
4. Transfer cells to 4 pre-chilled sterile round bottom centrifuge tubes (Falcon 2059)
5. Centrif. at 5000 RPM, 5 min, 4°C
6. Decant sup. and place on ice.
7. Re-susp. pellets into 5ml of sterile ice cold 20% trehalose or 10% glycerol, 2.5% sorbitol (control)
8. bring volume to 25ml in same solution.
9. Centrif. as above.
10. Immediately decant sup., place on ice.
11. Resusp. cells into 25ml of liquid as above

12. Repeat Centrif resusp. into 25ml of solution
13. Repeat Centrif as above, pipet out carefully the sup. Leave behind less than 100ml of sup.
14 Place tubes on ice.
15. Gently resusp. cells.
16. The Control cells can be aliquoted into tubes and frozen at -80°C (~50µl into pre chilled 1.5ml tubes)
17. Treated prepared cells will be used.

Cell Dry Down

1. 40ml aliquots of the lysate prepared cells are pipetted into metal cups and placed into shell freeze. Equilibrated at -40°C shell temp.
2. Following drying protocol used to dry down cells.

Product Name : comp cells
Product Number :
Operator Name : JJ

Freeze

Setpoint 1:	4	Time 1:	0
Setpoint 2:	0	Time 2:	0
Setpoint 3:	0	Time 3:	0
Setpoint 4:	0	Time 4:	0
Setpoint 5:	0	Time 5:	0
Setpoint 6:	0	Time 6:	0
Setpoint 7:	0	Time 7:	0
Setpoint 8:	0	Time 8:	0
Freeze Setpoint:	4		
Freeze Time:	0		
Vacuum Start Setpoint:	3000		

Primary

Setpoint 1:	4	Time 1:	600	Vacuum 1:	3000
Setpoint 2:	10	Time 2:	120	Vacuum 2:	3000
Setpoint 3:	10	Time 3:	480	Vacuum 3:	3000
Setpoint 4:	15	Time 4:	120	Vacuum 4:	3000
Setpoint 5:	15	Time 5:	480	Vacuum 5:	3000
Setpoint 6:	20	Time 6:	120	Vacuum 6:	3000
Setpoint 7:	20	Time 7:	480	Vacuum 7:	3000
Setpoint 8:	25	Time 8:	120	Vacuum 8:	3000
Setpoint 9:	25	Time 9:	480	Vacuum 9:	3000
Setpoint 10:	30	Time 10:	120	Vacuum 10:	3000
Setpoint 11:	0	Time 11:	0	Vacuum 11:	0
Setpoint 12:	0	Time 12:	0	Vacuum 12:	0
Setpoint 13:	0	Time 13:	0	Vacuum 13:	0
Setpoint 14:	0	Time 14:	0	Vacuum 14:	0
Setpoint 15:	0	Time 15:	0	Vacuum 15:	0
Setpoint 16:	0	Time 16:	0	Vacuum 16:	0

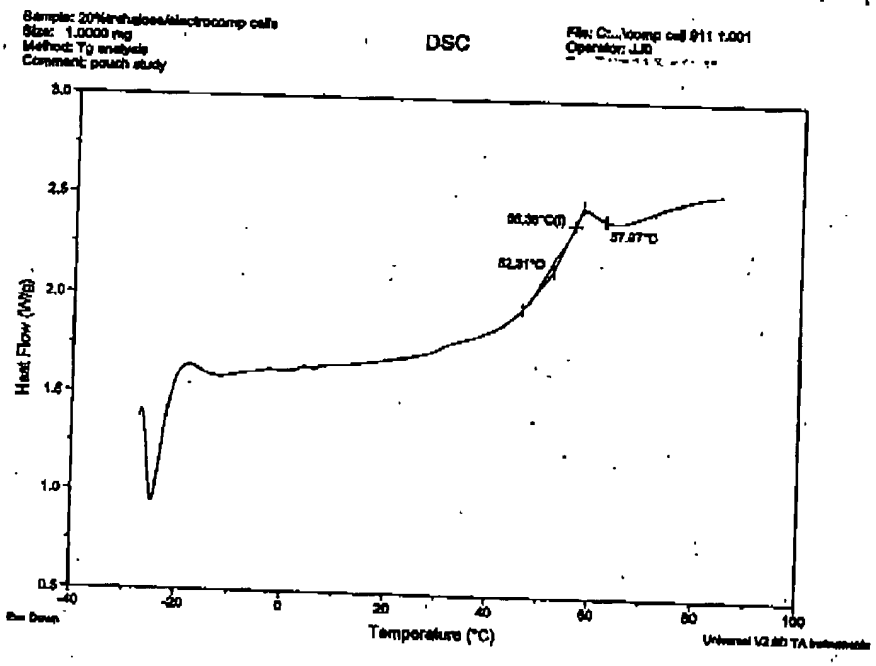
Secondary

Setpoint:	30
	600
Setpoint:	999
Setpoint:	30

Alarms

Condenser:	-40	Vacuum:	3000	Power:	10

After drying the cups are stored at RT in a desiccant chamber. Two dried cell discs are crushed into a DSC pan and a Tg value is determined.



Dried cell disc re-suspended in 4 ml of H_2O and plated to determine cell viability:

assume 10^{10} cells/ml

$\downarrow$ 1/1000

10^7 cells/ml → 1 ml in 100 ml LB plated, expect 10^5 cells

→ 10 ml in 90 ml LB plated, expect 10^6 cells

$\downarrow$ 1/1000

10^4 cells/ml → 100 ml plated, expect 1000 cells

1 ml in 10 ml of LB plated, expect 10 cells

10 ml in 90 ml LB plated, expect 100 cells

Cell viability determined by Alan G.

Dried cells were ~10% of control cells. The dried cells were electrocompetent.

Electrocomp. Cells
Fast dry down

1) Electrocomp. cells were prepared as above (p 48) except drying was done for 1h at 30C / 3000 mTorr.

2) Plating for survival and electrocomp. was done by Alan G.
Viability was close to control cells and electrocomp was better (~2 fold) for dried cells compared to control cells.

NOTEBOOK ENTRY C

Attia
Linda

Desiccation of XL10Gold (Chemicomp Maryland Strain)

1 Sunday night, started o/n cultures of our best selling chemicompetent strain, namely, XL10Gold (Cam R), 5ml x 2, in 20sq, in LB, 37°C shaker.

2 This AM, inoculated 1:100 (or maybe 1:85 dil) into fresh medium, and grew at 37°C (not 24°C) for ~ 3 hrs, to a final OD550 of 0.4, at which point, the flasks were removed (250ml flask, w/ ~100 mls of culture) onto ice.

3 Chilled on ice ~ 30'

4 Transferred ~ 40-45 ml each into precHilled Nalgene round-bottomed tubes, and spun down cells at 1600 rpm, for ~ 6 mins, with the brake set at ~ 5.

5 Immed after spin stopped, decanted sup, added to 1 set of samples, added (1/4 vol) FSB → and made competent for ~ 18 mins on ice

⊛ To the 2nd set of samples, did NOT add anything but pellets on ice (At end of incubation, added sauce vol (1/4) of 2x Trehalose

6 After the incubation, spun down cells as before.

7 Decanted as before, added 5% Trehalose to all samples to wash out traces of the FSB/etc which might hurt cells during desiccation.

8 Spun down cells as before — to pellet at end of spin, added

Karen Dolter

Contd:

Desiccation of X. c. 1b Gold — Design Expt 6.

- 1:40 (the starting volume) of Trehalose 20%. (Did not add Dextran to this prep), and quickly aliquoted x 150 µl each into the 2ml glass vials, all on ice.
- (Aliquoting took ~ 17-15 mins)
- For Frozen controls: FSB + (FSB/DMSO) → -80°C
- Dried at 3000 mTorrs, 30°C, for ~ 14-15 hrs.

(#) NOTE: Call maintenance guy for the Lyostar — the vacuum took more time (normally starts immediately, this time took 2 hrs, so ~ 4 mins later only vacuum actually started) than normal.

Plan of action for Transformation of the X. c. 1b Gold (Cam R) strain:

- Dilution Studies — (a) w/ FSB (or FSB-DMSO)
  OR (b) w/ 5% Trehalose soln
  OR (c) Peptone-H2O.
- If planning to use the rehydrated samples for both transf. efficiency studies (AS WELL AS post-desiccation viability studies) will need to use (a) - but to see if FSB/FSB-DMSO itself elicits higher cell death compared to other less harmful solns (like Trehalose soln), will need to include (a) and (b).

- Following final set of transf. studies done w/ above 30-40 seconds of heat shock at 42°C, try a VARIETY/RANGE of different Heat Shock periods, as well as higher (or lower) heat shock temperatures.
- Might need to use ~~Dextran~~ PVC at both 10 µg and 100 µg/100 µl of rehydrated cells — because cell death may be too high in the desiccated samples. With both TIME: 1 or 1½ hours?